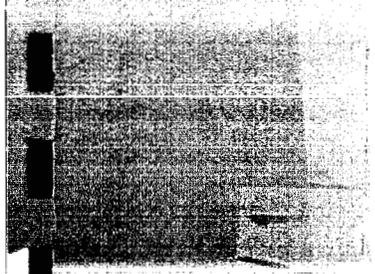


PHILCO

A SUBSIDIARY OF Ford Motor Company

AERONUTRONIC DIVISION

(NASA CR-55744; ~~Publication No.~~ U-2479)

OTS: $ 4.60 ph, $1.49 mf

0049502

Aeronutronic,
Newport Beach, Calif.

OTS

QUARTERLY ENGINEERING PROGRESS REPORT

STUDY OF BASIC BIO-ELECTROCHEMISTRY

Prepared for: National Aeronautics and Space Administration
Washington, D. C.

(NASA ~~Under~~ Contract NASw-655)

Reporting Period: 20 September ~~to~~ 19 December 1963

Prepared by: M. H. Boyer,
R. C. Bean,
Y. H. Inami, and
W. C. Shepherd

This work is jointly sponsored under NASA's Space Power Technology as well as Biotechnology and Human Research Programs.

19 January 1964

43 p refs

CONTENTS

ILLUSTRATIONS

SUMMARY

1. The anomolous electrical currents produced by the urea-urease system have been traced to increases in pH resulting from rapid urea hydrolysis and to the effect of the pH on oxidizable impurities in the urease preparations. The observations indicate that there is little possibility of any current arising from intermediate activated substrate or substrate-enzyme complexes.

2. Further study of the D-amino acid oxidase cell has made it clear that observed currents are derived from the electrochemical oxidation of aromatic pyruvate derivatives. Indole-3-pyruvate and p-hydroxyphenyl pyruvate are readily oxidized in the enol form due to their abilities to form quinones upon oxidation. The rate of the electrochemical oxidation is limited by the low concentration of the enol tautomer at the optimal pH for enzyme activity and by the slow rate of equilibration between the tautomers. These factors have interfered with efforts at coulometric determinations of electrochemical yields.

3. Further efforts toward detection of any direct electrochemical reaction of a reduced enzyme have been directed to the preparation of large quantities of crystalline D-amino acid oxidase and of a sulfite-nitrite reductase. Preparation of these enzymes in quantity will permit their use in the electrochemical cell at concentrations suitable for observation of direct participation in the electrode reaction.

4. Further studies of electrochemical activity of suspensions of intact micro-organisms and mitochondria confirmed the lack of activity in the absence of an intermediate electron carrier such as ferricyanide.

5. A new electrochemical cell has been fabricated which should give rise to much greater flexibility in enzyme tests.

6. An approach has been developed for the use of a variable synthetic molecule in studying the effect of size and shape of a macromolecular matrix upon the electrochemical characteristics of a specific redox prosthetic group. It is expected that information obtainable from such a study will be of considerable value in determining the feasibility of efficient enzyme-electrode interactions.

7. Theoretical considerations of the currents to be expected from efficient, direct enzyme-electrode reactions have been presented. Treatment of simple limiting case for an enzyme attached to an electrode in the form of a monomolecular layer, neglecting diffusional factors, indicates that maximal current densities on the order of 5.0 $\mu amp/cm^2$ for an oxidative enzyme of normal size and turnover number are obtainable.

SECTION 1

INTRODUCTION

The objectives of this program as outlined in previous quarterly reports have been to carry out a study of the basic processes involved in the generation of electrical energy by biological systems. The approach which was adopted was, first, to determine the classes of biological systems capable of electrochemical activity. These were then to be subjected to detailed study in order to define the specific reactions involved in the electrochemical process.

An associated objective was to study the more pragmatic aspects of the generation of electrical energy by living bacterial systems. Particularly, it was desired to examine factors such as theoretical efficiency of bioelectrodes, the performance of electrodes with attached vs unattached organism cultures, and the viability and growth of bacterial cultures in the presence of electrodes.

In accordance with these objectives the first two report periods, and part of the present period have been directed to study of the electrochemical activity of a number of enzyme systems including D-amino acid oxidase, urease and glucose oxidase, etc. Also included were studies on several bacterial systems, both attached to the electrode and suspended in solution, including B. subtilis, Proteus vulgaris, and suspensions of cell mitochondria. As a consequence of this work, the mechanisms of electrical energy production by amino acid oxidase and by urease have been traced

in considerable detail. Final results on these systems are presented in the present report. In these, and all other cases studied to date, the mechanism appears to involve formation of low molecular weight electroactive substances as intermediate or final products of the reaction between the biological agent and it substrate. It is these substances which provide the electrode reaction.

Recent thinking as to possible applications of bio-electrochemical phenomena, including the generation of practical quantities of electrical power, is, to an increasing degree, encountering the question of direct participation of biological macromolecules or bacterial cells in oxidation-reduction processes at electrodes. Accordingly, it has been decided that this program should place increased emphasis upon determination of whether or not such direct participation ever occurs.

The latter part of the present report period has been devoted primarily to this problem. Initial approach has been to define, on the basis of theoretical considerations, the conditions under which direct participation of biological molecules might be observable. Subsequent work will be concerned with attempts to observe such participation under the theoretically most promising conditions. A promising part of the program will be study of the electrochemical behavior of synthetic macromolecules which correspond to biological macromolecules in size and structure, but which have attached groups of known oxidation-reduction characteristics.

SECTION 2

METHODS

Procedures for measurements in the electrochemical cell have been described previously. In some of the recent experiments, particularly those involving coulometric yield, the Anatrol Potentiostat, rather than the manual potentiostat, has been used.

Bacterial growth for Proteus vulgaris and Bacillus subtilis were the same as previously described. E. coli cultures for preparation of sulfite-nitrite reductase were prepared by adapting strain B to growth on C medium (1), a medium containing only inorganic salts with glucose as carbon source. Sulfate provided the only source of sulfur in order to induce formation of the enzyme for sulfite reduction. After growing starter cultures in erlenmeyer flasks, an 8 liter fermenter was inoculated and bacteria harvested after 18 to 20 hours of growth at 37^{o}. The cells were collected in a continuous flow head of the Lourdes Betafuge and washed once with distilled water. The cells were dried by lyophilization after suspending in water and the dry cells stored until used for enzyme preparation or for electrochemical investigations. Enzyme preparative methods are indicated under the appropriate section in Results.

SECTION 3

ELECTROCHEMICAL STUDIES ON BIOLOGICAL SYSTEMS

3.1 UREA-UREASE SYSTEM

a. Experimental

The urea-urease system has presented a rather consistent dilemma during the earlier investigations on this program. With bright platinum electrodes, no oxidation of ammonia could be observed under the normal conditions of pH (6.5 to 9.0). This is evident in the comparison of the polarization curves for buffer alone (tris buffer = tris (hydroxymethyl) aminomethane, 0.1 M) at pH 9 and the same buffer containing 0.25 M ammonium nitrate (Figure 1). There is little significant difference in the two curves. Nevertheless, the action of urease on urea resulted in an augmented current in the electrochemical cell.

As demonstrated in the previous quarterly report, the urease preparations are responsible, in themselves, for a significant current. It was suspected that the current increase during the urea hydrolysis might be due to the effect of the reaction upon some of the components in the enzyme preparation rather than from reaction products. The experiments below were designed to test this hypothesis.

A polarization curve for a urease-urea hydrolysis is given in Figure 2. Here the reaction had been allowed to proceed for half an

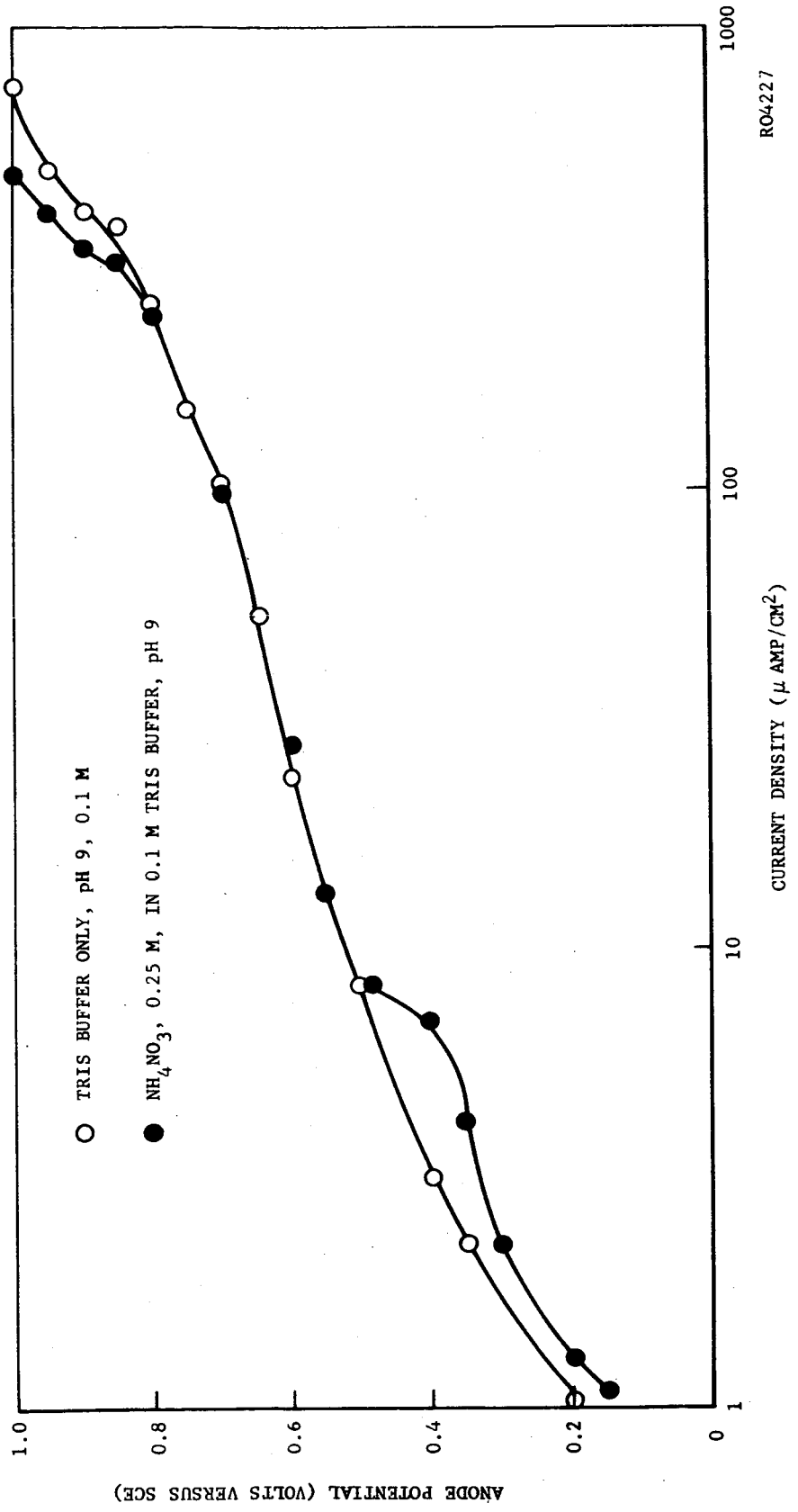


FIGURE 1. NH_4NO_3 POLARIZATION CURVE

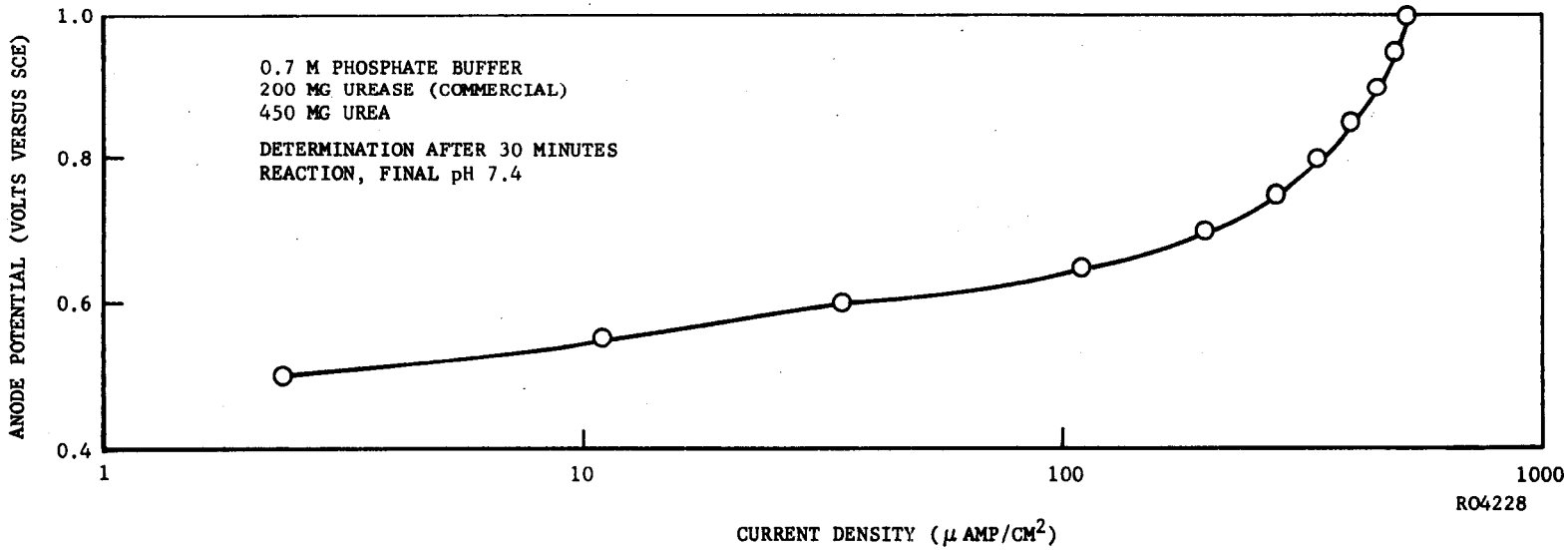


FIGURE 2. POLARIZATION CURVE FOR UREASE-UREA SYSTEM

hour which would give the optimal current, and almost complete hydrolysis. The reaction was carried out in 0.7 M phosphate buffer having an initial pH of 6.5 but at the time the curve was made the pH of the mixture had changed to pH 7.4. The curve shown corresponds closely to those given for pH 9 tris buffer.

The effect of dialysis upon the electrochemical behavior of commercial urease preparations is shown in Figure 3. The enzyme solution, 6.6 mg/ml, was dialysed against equal volumes of buffer and the dialyzing media as well as the dialysed enzyme were tested for electrochemical activity. It is apparent that a large amount of electrochemically active material, presumably of low molecular weight, was able to pass through the dialyzing membrane. Despite the loss of a great deal of oxidizable material, however, the background current from the enzyme remained high and the addition of urea to the enzyme caused the usual increase in current. Addition of urea to the dialyzing media had no effect on current output.

A comparison of extent of hydrolysis with the change in current is presented in Figure 4. The parallel of current with degree of hydrolysis is unmistakable.

Another parallel was found in the comparison of pH change with current production in Figure 5. Despite having a rather concentrated phosphate buffer in these experiments, i.e. 0.7 M, a large change in pH occurs and the current rises in proportion to the change in pH.

With urease alone, a deliberate addition of NaOH to the solution, sufficient to cause a pH change similar to that found in the normal reaction system, caused a similar rise in current (Figure 6).

Finally, the effect of a highly concentrated phosphate buffer was tested. Using a 2.5 M phosphate buffer, pH 6.5., no change in current was found during the reaction using the normal proportions of urea and urease.

CONDITIONS: UREASE, COMMERCIAL, 333 MG IN 50 ML. pH 6.5, 0.1 M PHOSPHATE BUFFER, DIALYZED AGAINST 50 ML BUFFER TWICE, 48 HR (A) AND 2.5 HRS (B) 30 ML OF DIALYZED ENZYME (U) OR DIALYZING MEDIA (A OR B) USED IN ELECTROCHEMICAL CELL TESTS. 450 MG UREA ADDED AT INDICATED POINTS. ANODE POTENTIAL 0.6 V VERSUS SCE.

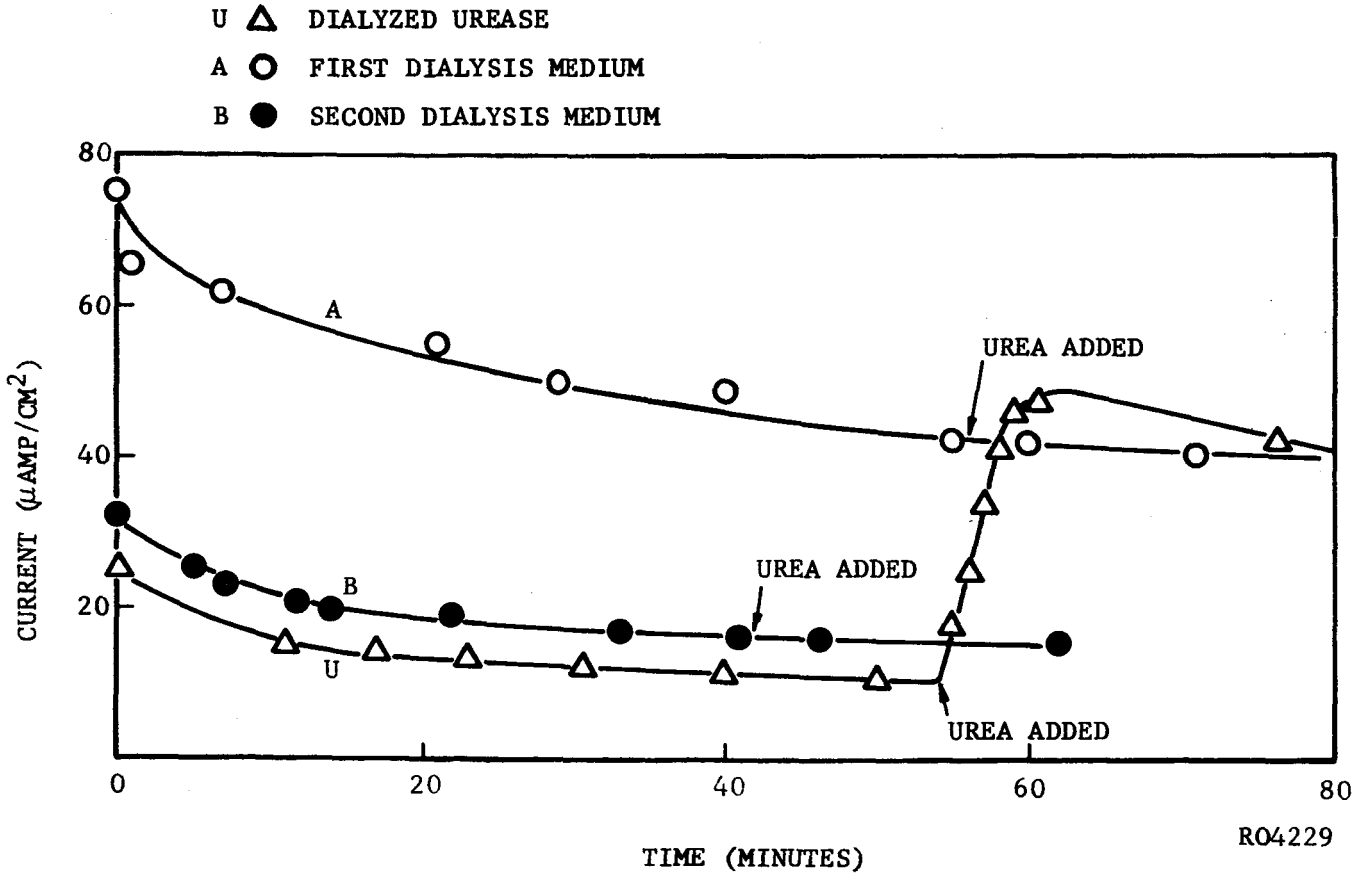


FIGURE 3. EFFECT OF DIALYSIS ON CURRENTS FROM COMMERCIAL UREASE

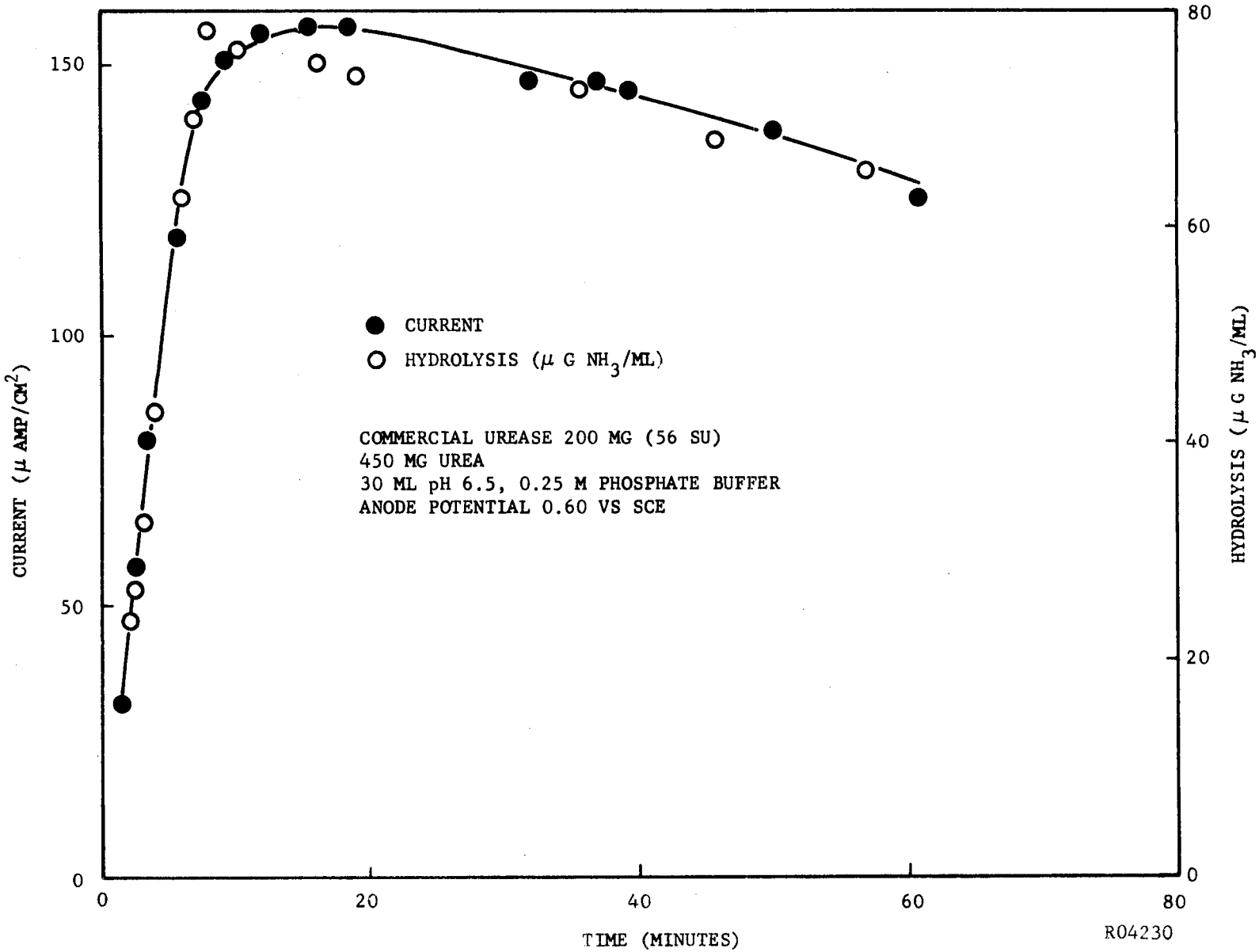


FIGURE 4. RELATION OF CELL CURRENT TO EXTENT OF UREA HYDROLYSIS

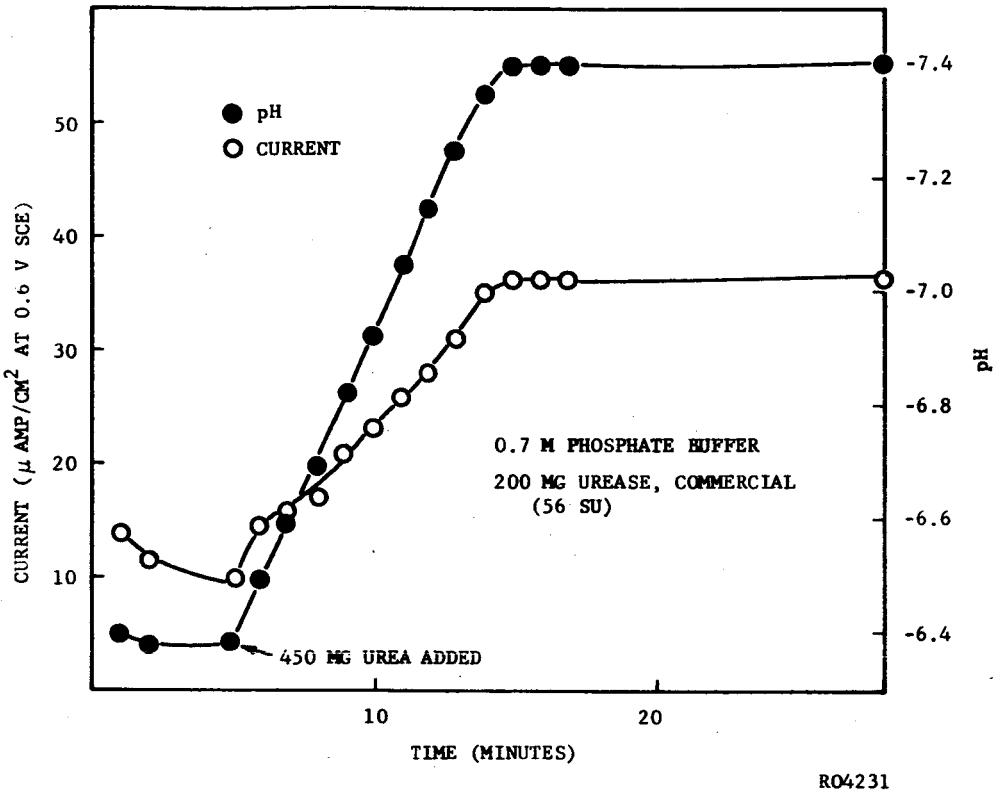


FIGURE 5. CHANGE IN CURRENT AND pH DURING UREA HYDROLYSIS

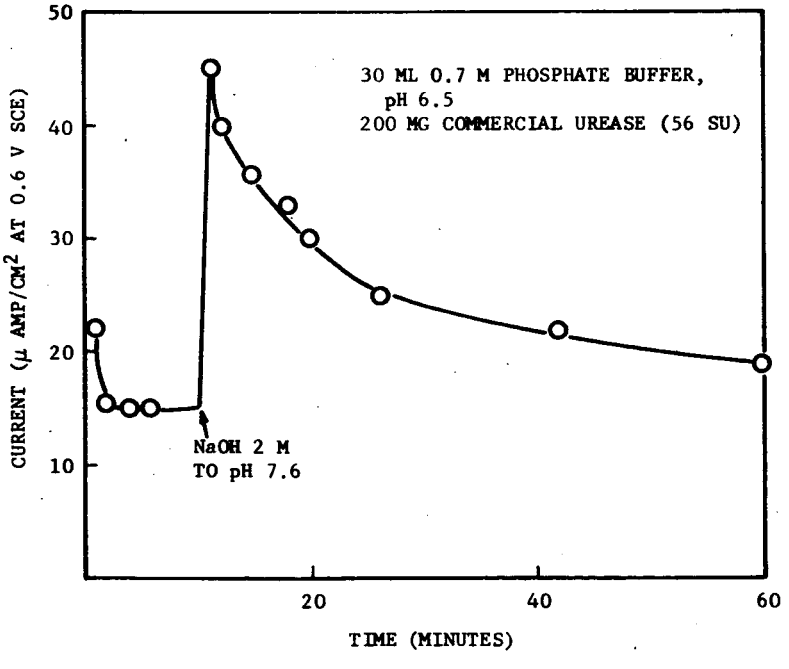


FIGURE 6. EFFECT OF pH ON UREASE CURRENT

As indicated previously, the purified crystalline urease prepared at Aeronutronic also showed electrochemical activity. Dialysis experiments indicated that the activity was due almost entirely to a low molecular weight material. The material responsible was probably 2-mercapto-ethanol, a reducing agent added to the preparations in the late stages of isolation to ensure complete activation of the enzyme. Tests of the mercaptoethanol demonstrated a high electrochemical activity and solutions of the enzyme which were aged in open vials to allow escape of volatile material soon lost any residual electrochemical activity.

b. Discussion

The evidence above makes it apparent that the basis for increased current during the action of urease upon urea in the electrochemical cell is the increased susceptibility to oxidation of material in the enzyme preparation with increasing pH. Rates of hydrolysis, increase in pH and current increase are all parallel. When pH is controlled with very strong buffers, no change in current occurs during the hydrolysis of urea by urease. Dialysis experiments have demonstrated the existence, in commercial urease preparations, of low molecular weight, electrochemically active material in substantial quantities. Aged, purified preparations of urease give rise to little or no current with or without the urea.

The source of the background current in the commercial urease is still not known although the background in fresh, crystalline urease is probably due to the presence of 2-mercaptoethanol which is used as an activator. A substantial part of the electrochemical activity of the commercial enzyme is contained in low molecular weight, dialyzable components but part of the electrochemical material is not removed from the protein by dialysis. If this current is due to oxidation of protein molecules, it still remains very dubious that the urease, itself, is responsible for the current noted. The active urease, in the commerical material, constitutes less than one hundredth of the total material present

and would not be likely to provide a substantial output. In any event, use of commercial urease preparations in high concentrations in close proximity to the anode in a biofuel cell would undoubtedly give rise to spurious currents over a fairly long period of time.

3.2 THE D-AMINO ACID OXIDASE SYSTEM

In the previous quarterly report, it was shown that electrochemical activity by this system is a consequence of the production of pyruvic acid derivatives from certain (aromatic) amino acids. It was also shown that these products can exist in two tautomeric forms of greatly different electrochemical behavior. Some additional studies of this system have been carried out during this report period, to further clarify the nature of the tautomeric transformation.

The three aromatic pyruvates, IPA, HPA and phenylpyruvate (PPA) all have structures which should favor a stable enol form as proposed for IPA in the previous report. Accordingly, spectrophotometric examination of HPA and PPA revealed behavior very similar to that of IPA. The changes in absorption spectra shown in Figures 8, 9 and 10 are consistent with the interpretation that the pyruvate exists in the enol form in the crystalline state or in organic solvents and as the keto structure in aqueous systems, particularly in basic solution.

It is therefore concluded that the same type of tautomeric relations exist for the three aromatic pyruvic acids, IPA, HPA and PPA. However, only IPA and HPA appear to have a form which is highly active electrochemically. Therefore, it cannot be the mere existence of the enol structure (or related form) which permits the oxidation of the aromatic pyruvate. Rather it must be that the enol form labilizes another part of the molecule so that oxidation becomes possible. The only difference between PPA and HPA is the existence of the phenolic group in the latter. Thus, it is easy to visualize a mechanism whereby quinoid structures may arise readily during oxidation of the HPA but not so readily in the PPA,

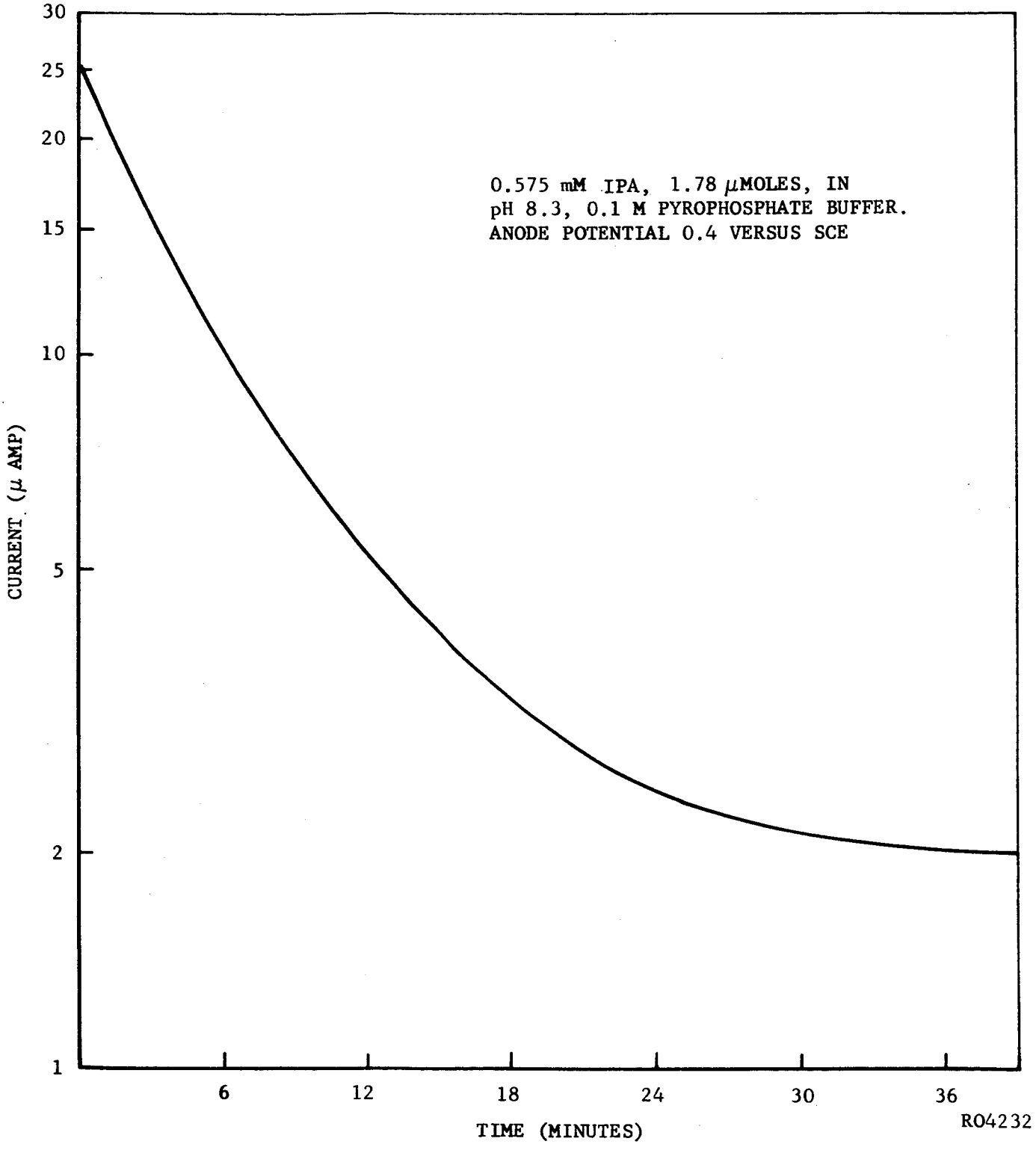


FIGURE 7. COULOMETRIC OXIDATION OF INDULE-3-PYRUVATE

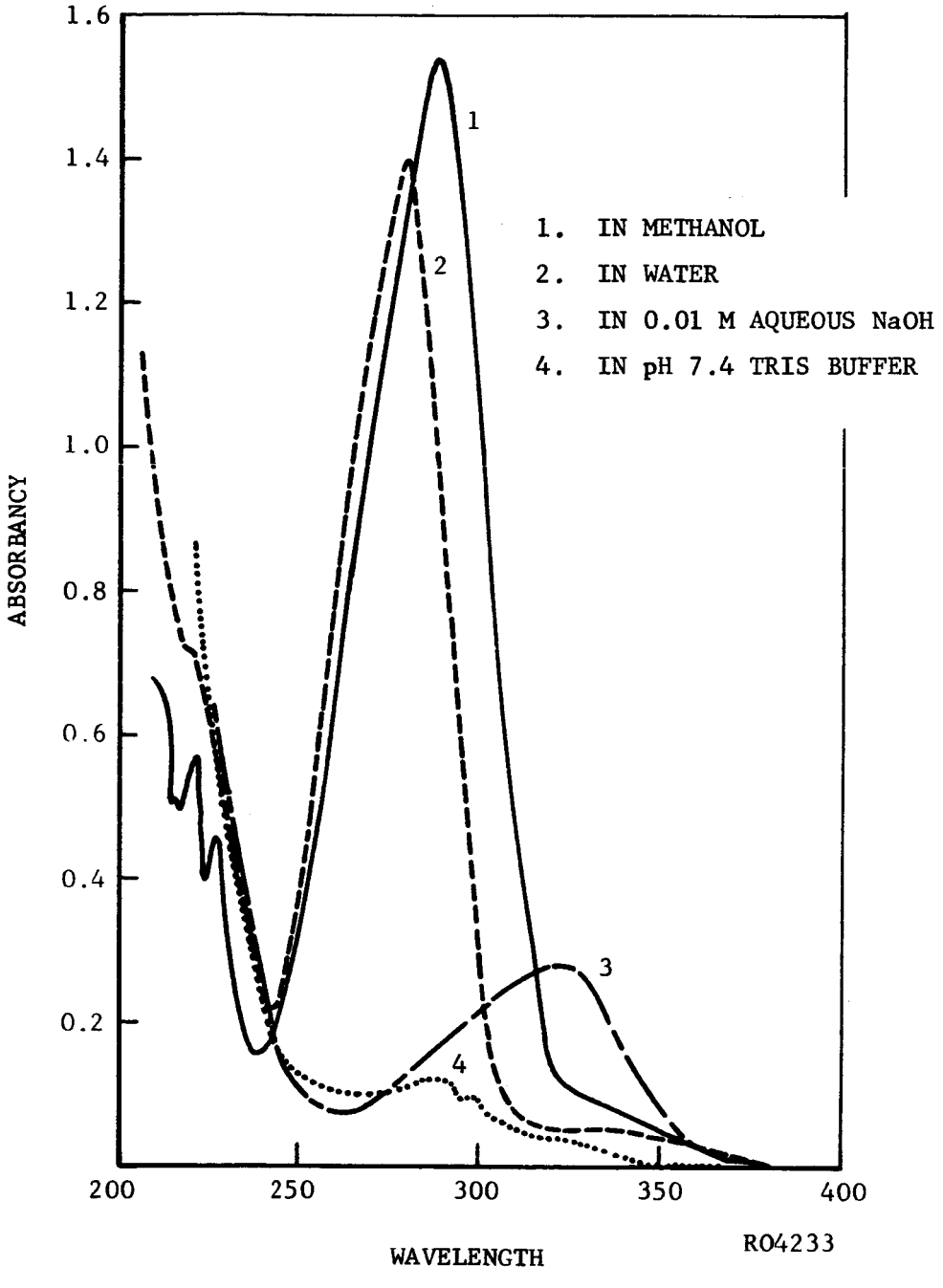


FIGURE 8. SPECTRA OF PHENYL PYRUVIC ACID IN VARIOUS SOLVENTS

A. HPA IN METHANOL, 8.66 μG/ML. NO CHANGE IN SPECTRUM WITH TIME.

B. HPA IN WATER, 8.66 μG/ML. SEE FIGURE 10 FOR CHANGE WITH TIME.

C. HPA IN, 8.56 μG/ML, IN 0.01 M SULFURIC ACID. LITTLE OR NO CHANGE WITH TIME THE CURVE FOR HPA IN METHANOLIC SULFURIC ACID WAS ALMOST IDENTICAL.

D. HPA, 8.56 μG/ML, IN AQUEOUS, 0.01 M NaOH. SLOW CHANGE WITH TIME, SHIFTING PEAK AND CHANGING INTENSITY. SIMILAR CURVE FOR METHANOLIC NaOH WITH PEAK AT 327 Mμ

E. HPA, 8.6 μG/ML, in pH 8.3, 0.1 M PYROPHOSPHATE BUFFER, 20 MINUTES AFTER INITIAL SOLUTION OF HPA.

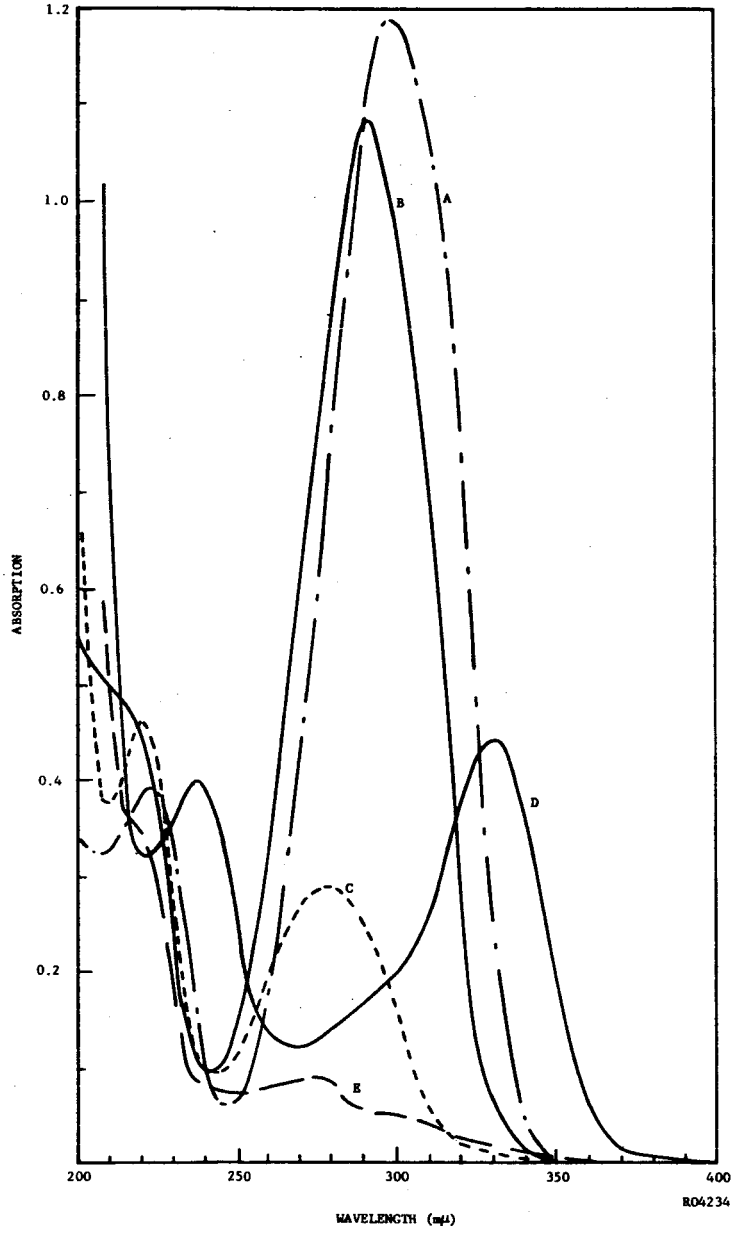


FIGURE 9. SPECTRA OF p-HYDROXYPHENYL PURUVIC ACID

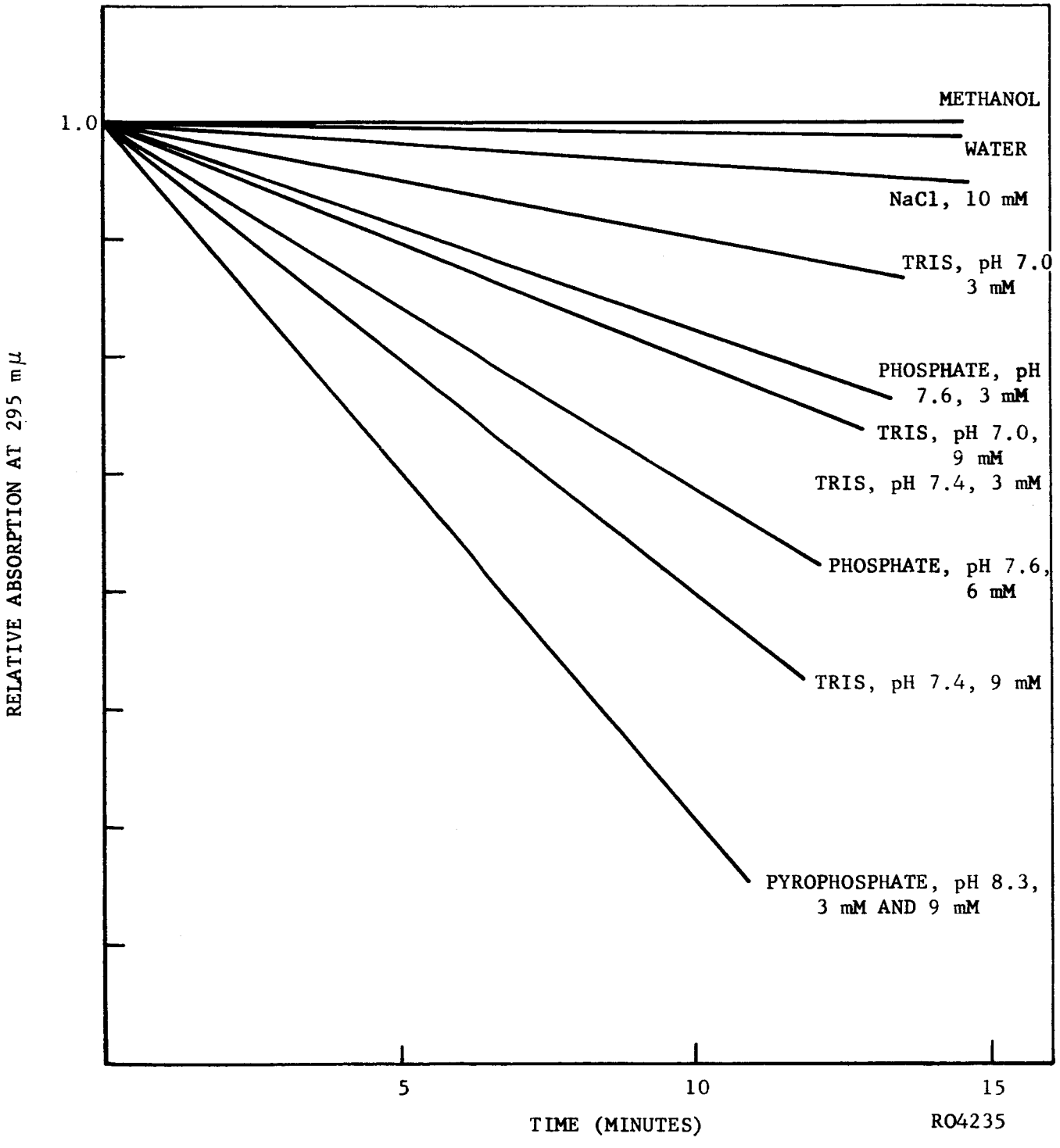


FIGURE 10. RATE OF CHANGE OF HPA ABSORPTION SPECTRUM AS AFFECTED BY IONS AND pH

lacking the phenol group. IPA would also be capable of forming quinoid structures readily. It would seem probable, if the participation of quinones were involved, that the anodic reaction may actually be oxidizing the ring structure of the pyruvates.

Some additional data showing the effect of various ions and pH values upon the rate of transformation from enol to keto form is shown in Figure 10. Rates are affected by pH, ion concentration and, to a lesser degree, by the specific ion used at a given pH and concentration.

The effect of the tautomeric shift upon electrochemical activity is shown by a plot of cell current vs time. Such a curve for indole-3-pyruvic acid (IPA) is shown in Figure 7. Although a high initial current is obtained upon adding the solid IPA to the buffer, the shift to the less active tautomeric form rapidly reduces the output of the cell. It was possible to demonstrate that the current, at a given time after dissolving the IPA crystals, was almost identical whether the solution had been subject to continual electrochemical oxidation or it had been standing idle during the same period. The failure to obtain a straight line curve for log current vs. time indicates that the electrode reaction is not limited simply by diffusion and concentration of IPA. Thus, it is apparent that the rate of the tautomeric shift and the low concentration of the electrochemically active form are limiting the rate of the electrochemical reaction rather than diffusion. A similar relation is found with p-hydroxyphenyl pyruvate (HPA). At the optimal pH for the enzyme reaction, the equilibrium in the tautomeric reaction for both IPA and HPA is far in favor of the tautomer of low electrochemical activity.

3.3 NITRITE AND SULFITE REDUCTASE SYSTEMS

a. Introduction

The reduction of sulfate to hydrogen sulfide by the organism, Desulfo-vibrio desulfuricans, or by related bacteria, has already been applied to biochemical fuel cells with a good degree of success (3).

Thus, it is of some interest to obtain more definitive data on the reactions involved in the reduction sequence and the effect of various intermediate reactions on the cell output. The possibility of obtaining a more efficient electrochemical reaction by interruption of the biological reduction at an intermediate stage is of interest. The hydrogen sulfide produced in the sulfate reduction is reoxidized anodically to sulfur which is not readily used in further reactions. Anodic oxidation of an intermediate product might result in regeneration of the original substrate (sulfite) which would allow a cyclic process to occur, i.e. reduction of sulfite enzymically with the utilization of organic material and electrochemical reoxidation with the production of useful energy. Similarly, some organisms reduce nitrate or nitrite to ammonia which may be reoxidized electrochemically but the anodic reaction requires such stringent conditions that they are not conducive to maintenance of the biological system. Interruption of the reaction at an intermediate state in such a reaction might allow a cyclic process, as above for sulfite, and a much better electrochemical yield. A possible intermediate is hydroxylamine which is oxidized much more readily under physiological conditions than the final product, ammonia.

Recently, it has become possible to prepare relatively pure enzymes or enzyme systems for the reduction of sulfite to hydrogen sulfide or nitrite to ammonia.(4,5). Enzymes for nitrite reduction have been prepared from many sources but that from Escherischia coli also reduces sulfite in the same manner that it attacks nitrite. It should be emphasized that a single enzyme is apparently responsible for reduction of both sulfite and nitrite as well as the reduction of hydroxylamine to ammonia. Thus, the enzyme preparation carries out the following reactions:

$$NO_2^- + 3NADPH_2 \longrightarrow NH_3 + 3NADP$$

$$HSO_3^- + 3NADPH_2 \longrightarrow H_2S + 3NADP$$

$$NH_2OH + NADPH_2 \longrightarrow NH_3 + NADP$$

Although hydroxylamine is also reduced by the enzyme, the evidence indicates that free hydroxylamine is not formed during the reaction with nitrite and that the complete six electron exchange occurs once the sulfite or nitrite becomes attached to the enzyme without release of intermediate products. The preparations from E. coli also have a cytochrome reductase activity which appears to be directly associated with the enzyme responsible for the sulfite and nitrite reduction. The physiological function of the enzyme in the bacterium appears to be for production of sulfur at the sulfhydryl level in a medium lacking in reduced sulfur since it is produced in response to a medium containing sulfate but lacking in reduced sulfur.

b. Experimental

Isolation of the sulfite-nitrite reductase of E. coli has been accomplished through a modification of the technique of Lazzarini and Atkinson.(6) Our procedure differs from that of the original authors in the use of lyophilized, dry E. coli and an initial grinding in the dry state rather than disintegration in a Hughes press. Activities and yields have been quite comparable with those in the original literature. Unfortunately, yields are low, even at best, when the requirements for use in the electrochemical cell are considered. Therefore, a large amount of material must be processed before sufficient high activity enzyme will be available for extensive and definitive electrochemical studies. The preparation of the sulfite reductase requires the growth of large quantities of bacteria and the growth has been carried out in an eight liter fermenter which gives overnight yields of about 20 g of wet cell paste. This is then lyophilized to give about 7 g of a stable, dry cell preparation which may be stored for a long period until required for enzyme preparation.

For enzyme isolation, 5 g of dry cells were ground in a cold mortar and suspended in 150 ml 0.04 of potassium phosphate buffer. Ribonuclease, 6 mg (crystalline, bovine, 43.5 Kunitz units/mg) and

deoxyribonuclease (pancreatic, 32000 Dorner units/mg) were added and the suspension incubated for 30 minutes, titrating occasionally with 1 M Tris buffer, pH 8.7 to maintain the suspension at pH 7.5. After centrifuging for ten minutes at 5000 x G to remove cell debris, the supernatant (145 ml) was treated with 0.5 volumes of saturated ammonium sulfate. The precipitate was removed by centrifugation and the enzyme precipitated by adding an additional 0.5 volumes saturated ammonium sulphate. After redissolving the enzyme in 20 ml 0.04 M, pH 7.4 phosphate a second precipitation between 0.35 and 0.5% ammonium sulfate saturation was carried out. This precipitate was redissolved in 20 ml buffer and dialyzed overnight against eight liters of 0.005M, pH 7.4 buffer containing 0.001 M cysteine. The enzyme solution was then adjusted to pH 6.0 with acetic acid and this precipitate removed prior to precipitation of the enzyme by adjusting to pH 5.0. This final precipitate will be used in the electrochemical studies. The final preparation causes oxidation of 1.7 moles of NADPH per hour per mg protein with a total of about 15 mg protein. Early preparations of the enzyme, from relatively fresh powder, contained a rather active diaphorase activity which interfered with the desired activity by competing for the reduced coenzyme. Subsequent preparations, using aged dry cells have contained only low diaphorase activities. The freshly prepared crude enzymes also have associated with them a nitrite reductase which is specific for nicotinamide adenine dinucleotide (NAD or DPN) rather than the nicotinamide adenine dinucleotide phosphate (NADP or TPN) required by the nitrite-sulfite reductase. The former enzyme appears to be unstable to storage and its activity diminishes to a very low value during a few weeks storage at freezing temperatures while the activity of the desired enzyme is maintained.

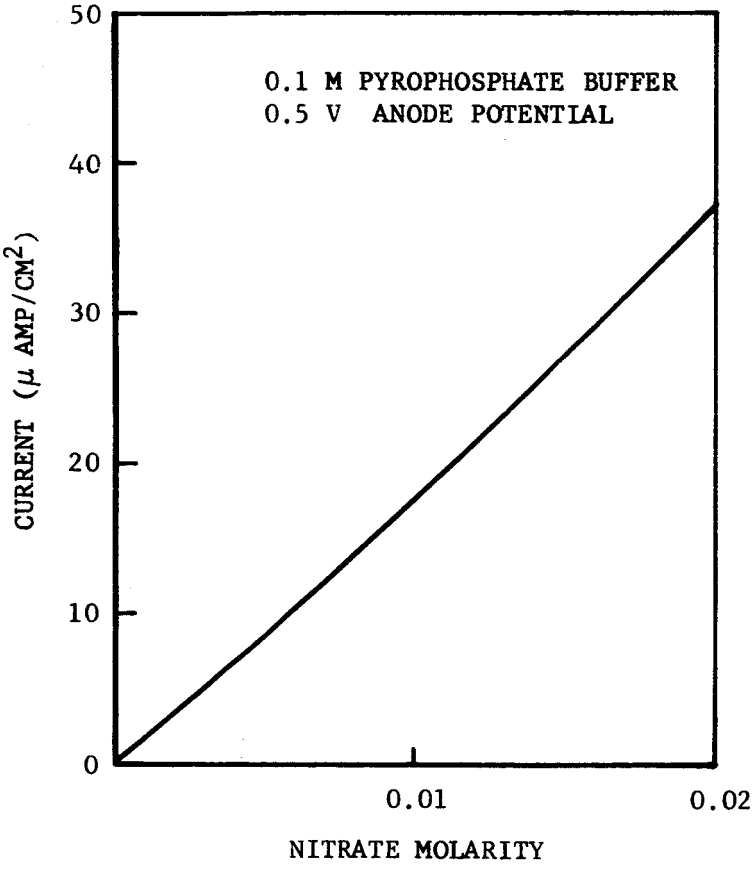
c. Discussion

In connection with the work on the sulfite-nitrite reductase, studies on the electrochemical characteristics of substrates and intermediates are being made. The rate of electrochemical oxidation of nitrite

as a functionof nitrite concentration is given in Figure 11. Characteristics of sulfite are given in Figure 12. Oxidation rates, in both instances, are low at reasonable anodic polarization. Hydroxylamine, on the other hand, shows a very effective anodic reaction. Data obtained from a coulometric yield determination (Figure 13) makes it apparent that multiple reactions exist in agreement with the observations of previous workers.(6,7) The first part of the curve, prior to the break, indicates that 3.75 electrons have been exchanged per molecule of hydroxylamine while another 0.35 electrons are obtained from the part after the break. This may be compared with the data of Lingane and Jones (6) who found a yield of 2.7 electrons and Davis (7) who obtained variable results of 2.0 to 4.6 for n. Both of these authors concluded that the products were complex. In the former instance, the major product appeared to be nitrous oxide (lost as a gas) and nitrate with a small amount of nitrite being formed. Davis assumed the major product to be nitrate. In both cases the potentials being used for theanodic oxidation were equal to or greater than 1.0 volts so that oxidation of nitrite to nitrate might be expected. In the present experiments, much lower potentials have been used and it seems reasonable to assume that the first part of the curve represents mainly the oxidation of hydroxylamine to nitrite (four electrons) while the slow oxidation evident later is the oxidation of nitrite to nitrate. Loss of a certain amount of nitrous oxide during the reaction could account for the failure to obtain the theoretical four electron exchange during the early part of the reaction.

3.4 FERRIDOXIN SYSTEMS

Ferridoxin is a recently discovered enzyme or coenzyme.(8) It is a low molecular weight protein (12,000) containing about ten atoms of iron per molecule. It acts as a biological electron carrier in photosynthesis (9,10) and in bacterial reactions involving hydrogenase.(8,11) It may be isolated from plants (10) or, more conveniently and in larger



RO4236

FIGURE 11. ANODIC OXIDATION OF NITRITE

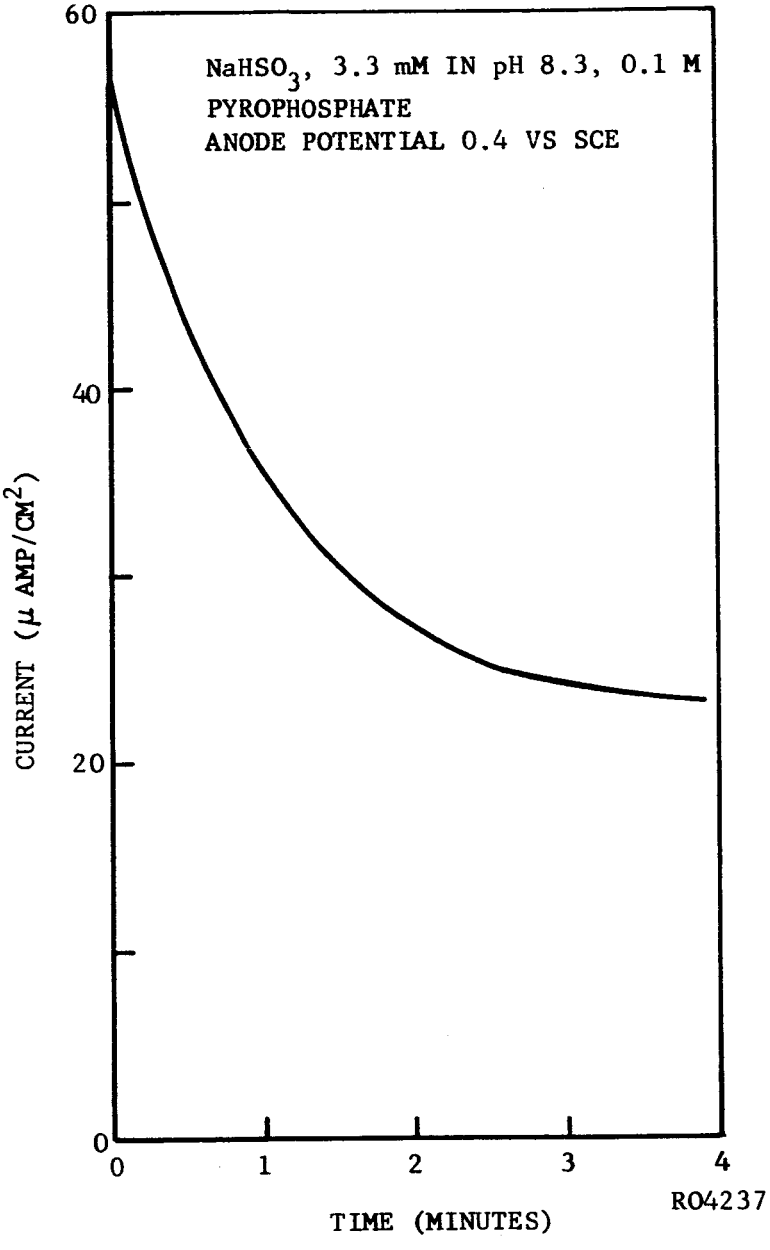


FIGURE 12. ANODIC OXIDATION OF SULFITE

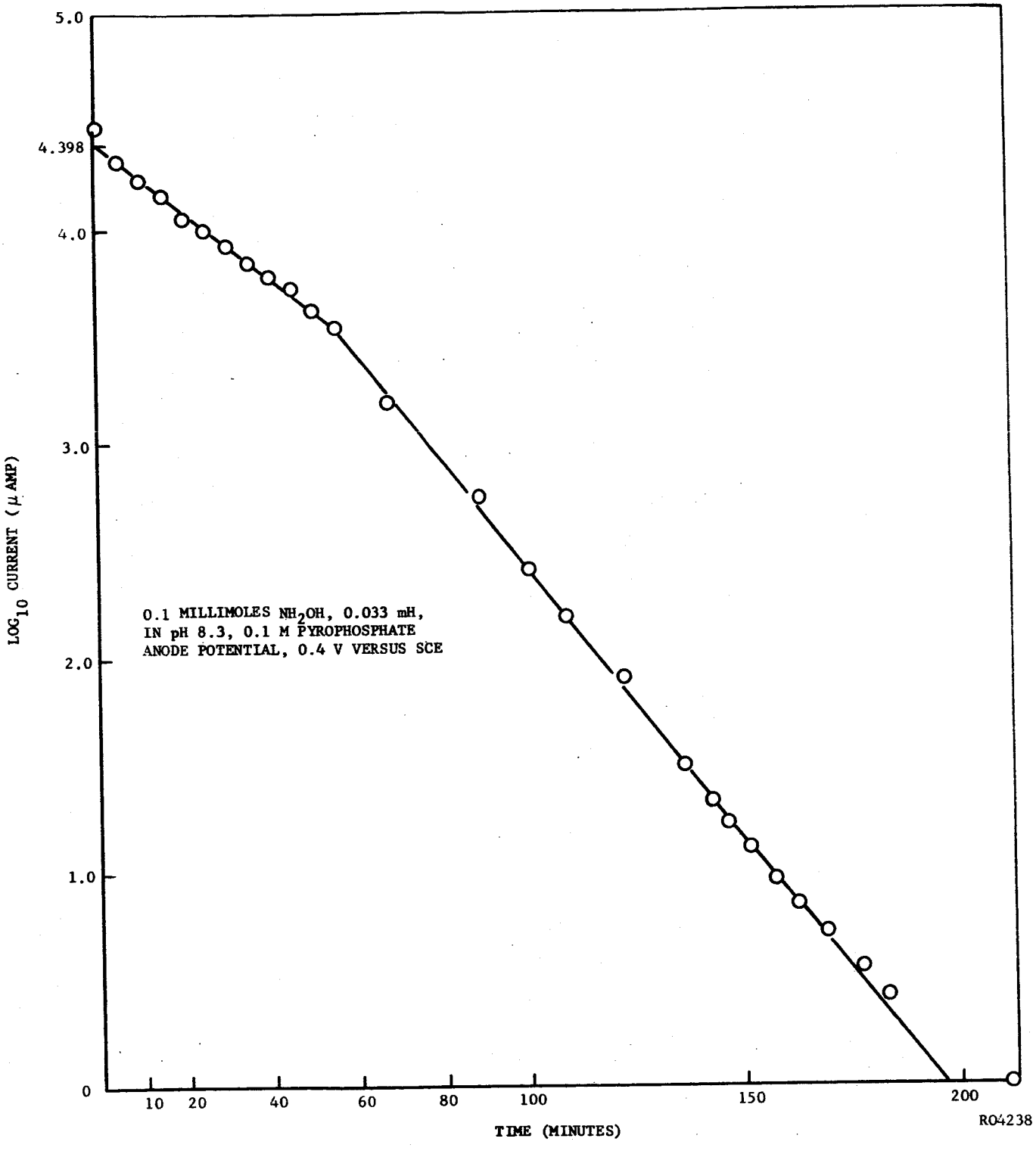


FIGURE 13. COULOMETRIC OXIDATION OF HYDROXYLAMINE

yields, from obligately anaerobic bacteria, particularly the nitrogen fixing Clostridia(1). It is unique in having the highest electronegativity of any biological electron carrier so far discovered, with E_o' about -415 to -430 mv at pH 7. Thus, it could be a promising candidate as a biological intermediary in a direct enzyme reaction with an electrode.

Several attempts have been made to prepare adequate quantities of ferridoxin from spinach leaves for testing in electrochemical applications. In these experiments it was necessary to follow the rather incomplete description of isolationgiven by Tagawa and Arnon (10) and the preparations have not been eminently successful. Ferridoxin has not been obtained in reasonably pure form nor in sufficient quantities for testing in the electrochemical cell. Further efforts on ferridoxin have been postponed pending receipt of a culture of Clostridium pasteurianum, a much richer source of ferridoxin.

Once obtained, it is proposed that reduced ferridoxin by prepared by a photosynthetic reaction with chloroplasts and tested in the electrochemical cell for a direct reaction with the electrode.

3.5 ELECTROCHEMICAL ACTIVITY OF INTACT ORGANISMS

a. Introduction

In preparation for electrode attachment studies, various bacteria have been grown and tested for electrochemical activity in free solution. Additionally rat liver mitochondria were retested to confirm earlier tests which indicated total lack of current production in absence of low molecular weight electron carriers.

b. Mitochondria

Rat liver mitochondria were prepared as previously (see first quarterly report) and tested for electrochemical activity using 0.045 M succinate as substrate. Either alone, or in presence of cytochrome C as carrier, no significant current was available. Background current was initially 10 to 15 amp (0.4 v SCE) and the

mitochondria alone, as well as with cytochrome C, gave 5 to 13 μamp. Addition of potassium ferricyanide (0.005M) gave rise to a current of 120 $\mu amp/cm^2$ which was limited by ferricyanide. Doubling the ferricyanide resulted in 180 $\mu amp/cm^2$ current. These results were comparable with those obtained previously. It must be concluded that cytochrome C at concentrations used, is not a highly efficient carrier. However, it must be emphasized that the concentration used, 0.4 mg/ml, amounts to approximately $6x10^{-7}_M$ which compares with $5x10^{-3}_M$ used for ferricyanide.

c. Bacillus subtilis

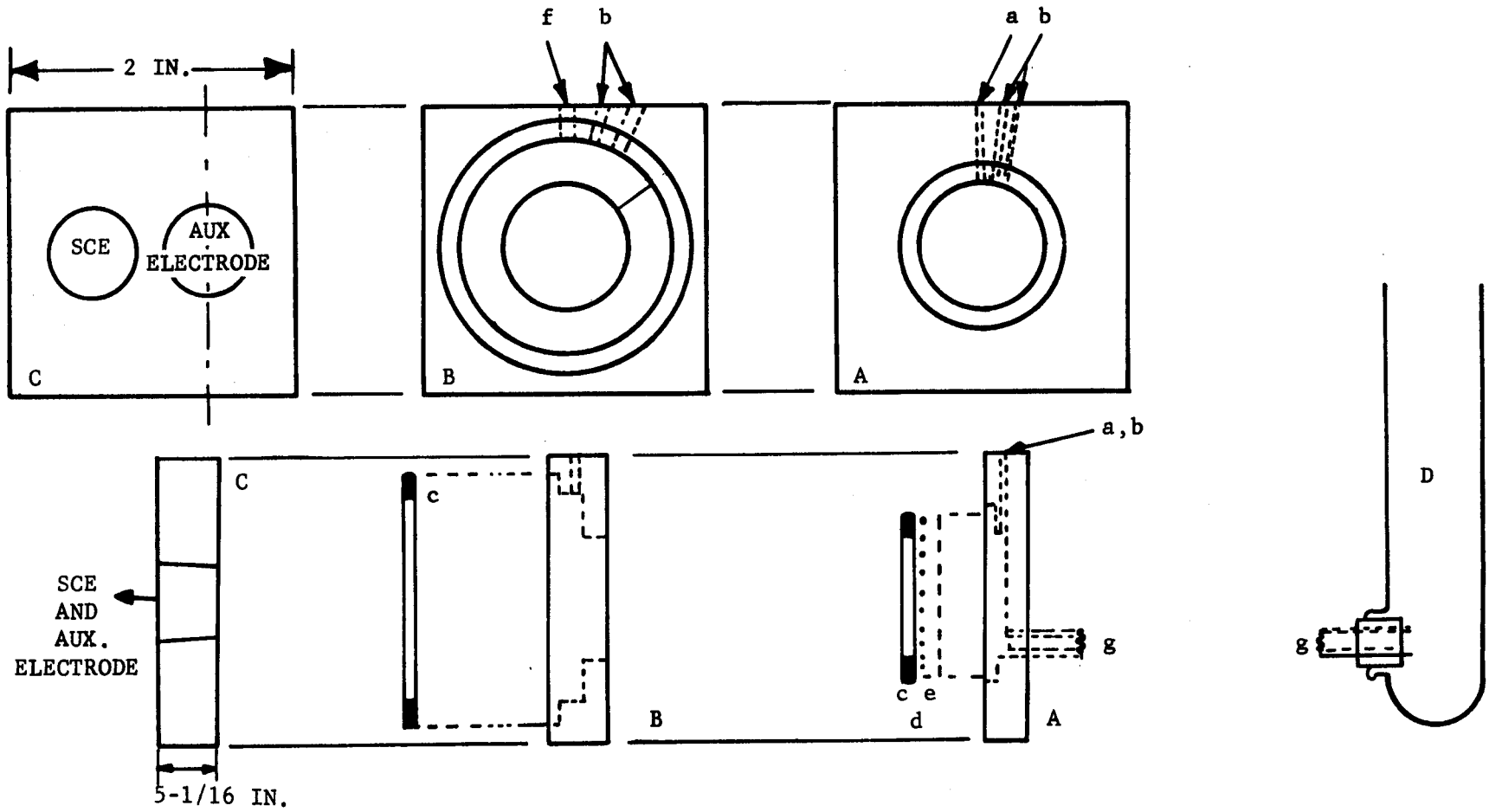
Cell suspensions of up to $12x10^9$ cells/ml produced an aerobic current which decreased from an initial 28 μamp to 5 μamp (0.4 v SCE) at the end of ten minutes. Addition of glucose to the suspension did not improve output. The decreasing initial current probably indicates that a minor component of the original suspension, rather than the bacterial activity, is responsible for the current found.

d. Proteus vulgaris

This bacterium is of interest since it forms coatings readily on metal surfaces. However, cell suspensions of $8x10^9$ cells/ml gave steady state currents of only 3 to 5 $\mu amp/cm^2$ (0.4 v SCE) even with glucose and/or nitrate or urea as substrates.

3.6 DESIGN AND FABRICATION OF AN ISOLATED ELECTRODE COMPARTMENT MICRO-ELECTROCHEMICAL CELL

A cell has been designed and built according to the configuration indicated in Figure 14. The objective of using this type of cell is to be able to have highly concentrated enzyme in the vicinity of the electrode without need for use of large quantities of enzyme and to provide a model system for study of systems analogous to enzymes or organisms attached directly to the electrode. The design also provides a compact arrangement which should allow normal electrochemical reactions to be carried out in much smaller volumes than must normally be used (5 to 6 ml total volume) with consequent savings on scarce material.



A - ELECTRODE COMPARTMENT UNIT
- a. ELECTRODE SHAFT APERTURE
- b. FILLING AND GASSING APERTURES
- c. SILICONE GASKET
- d. SUPPORT SCREEN
- e. SEMIPERMEABLE MEMBRANE

B - FUEL CHAMBER
- f. STIRRER APERTURE

C - BACK PLATE

D - SCE TUBE
- g. TEFLON CONNECTING TUBE WITH SALT-AGAR BRIDGE

RO4239

FIGURE 14. MICROELECTROCHEMICAL CELL WITH SEPARATE ELECTRODE COMPARTMENT

The present cell has been fabricated from nylon sheet, using silicone rubber gaskets. The vibrator motor for the electrode and for the main compartment stirrer is a modified electric razor (Ronson) with vibrational amplitude controlled by a variable transformer.

Preliminary tests are being carried out upon the use of the new cell with normal systems prior to use with a membrane separator for the electrode compartment. Initially, a high background current has been obtained in the new cell, even with buffer alone and means are being sought to reduce this background level to a normal minimum. The source of current may be material adsorbed on the nylon material used in the body.

SECTION 4

USE OF MODEL MOLECULAR SYSTEMS IN THE STUDY OF THE REACTION OF BIOLOGICAL MACROMOLECULES AT ELECTRODES

The purpose of the micro cell described in Section 3.6 is to provide optimum conditions under which to examine the existence of a direct reaction between the electrode and reduced enzyme. This cell provides a region, in close proximity to the electrode surfaces, wherein the enzyme concentration may be increased to a level commensurate with molar concentrations used in normal electrochemical reactions. In a diffusion controlled process involving an enzyme as the diffusing species, one would expect steady state currents of a much lower magnitude than for a smaller molecule at a similar molar concentration, due to the difference in diffusivity. It follows that even where no kinetic complications exist, currents of detectable magnitude cannot be expected from a protein-electrode interaction unless the enzyme concentration in the immediate environs of the electrode is of the order of 10^{-4} to 10^{-3} M. For DAO, 115 mg/ml would provide a concentration of 10^{-3} M. If contaminated with other inactive protein, correspondingly greater quantities of preparation would be required. Since such quantities of pure enzyme are exceedingly expensive and difficult to obtain. The isolated electrode cell has been developed to provide a means for using very small volumes (0.25 ml) of concentrated enzyme solution or suspension in the neighborhood of the electrode while providing an adequate source of substrate in the membrane-separated main compartment.

The question, however, is not only that of adequate concentration. Steric and orientation effects of the large protein molecule may play a very large role in the current carrying capabilities of a redox enzyme. Other avenues must be explored in order to test whether such factors, rather than diffusion, may be controlling. One possibility is the use of synthetic models for redox enzymes. Such models would consist of molecules of varying dimensions containing specifically attached redox groups of known characteristics. Through chemical or electrochemical reduction and subsequent oxidation in the electrochemical cell, it would be possible to determine how increases in molecular size and type of matrix molecule would affect the behavior of the redox group.

Redox polymers, such as those prepared from dihydroxybenzene (12) or the polythiol styrenes (13) might be used as enzyme models but these polymers as presently available suffer from disadvantages of low aqueous solubility, lack of uniformity of chain length and an excessive number of redox groups per macromolecule in comparison with the enzyme system. A better approach to a model enzyme system might be obtained from the specific coupling of a redox dye to a peptide or protein to form a redox group analogous to that of the prosthetic group of the enzyme. Such coupling may be carried out through use of techniques similar to those of Landsteiner (14) for the incorporation of hapten groups into protein antigens. The diazonium salt of a redox dye (e.g., toluylene blue, which contains primary amine groups not required for the reversible oxidation) may be coupled with the tyrosine or histidine residues in peptides and proteins of varying dimensions and chain lengths. Similarly, it may be possible that phenolic redox dyes could be coupled through the reverse procedure of diazotization of an aromatic amine group attached to the protein. Coupling of other redox dyes could also be accomplished through mild esterification procedures.

It is presently planned to prepare several such derivatives for electrochemical tests. First efforts will be directed toward the coupling of toluylene blue (E_o 0.601, E_{m7} 0.115) with tyrosine; a tripeptide containing tyrosine (tyrosyl-glycyl-glycine); a naturally occurring small peptide (e.g. tyrocidin, an antibiotic, decapeptide containing one tyrosine residue); a small protein (e.g., lima bean trypsin inhibitor or ribonuclease, m.w. 12,000 and 17,000, respectively); and a medium size protein (hemoglobin, β-lactoglobulin, ovalbumin or serum albumin). The various derivaties should be tested for chemical reactivity with mild oxidants and reductants to determine equivalent oxidation numbers. Electrochemical activity will be determined after a chemical reduction and separation of the chemical agents from the reduced model. Attempts will be made to correlate rates of electrochemical oxidation with the size of the matrix molecule, diffusion rates, shape, steric and orientation factors in the various models.

SECTION 5

THEORETICAL CONSIDERATIONS RELATING TO THE DIRECT OXIDATION-REDUCTION OF BIOLOGICAL MACROMOLECULES AT ELECTRODES

Any electrochemical reaction can be represented in terms of six general steps, as follows:

1. Diffusion of reactants to the surface
2. Adsorption of reactants onto surface
3. Reaction in adsorbed phase
4. Electron transfer between one or more adsorbed species and the electrode
5. Desorption of products
6. Diffusion of products away from electrode

Kinetic behavior is determined by the relative rates of these different steps. Simplest behavior is observed when one is much slower than all of the rest. In some instances one or more of the six steps may not be pertinent.

Since the steps of the scheme take place in sequence, if a maximum rate for any one is known, it defines a maximum rate for the system. The other steps could lead to lower overall rates, but never to higher ones. However, at any given electrode, two or more such reaction schemes may operate simultaneously and in such cases the total rate will be represented by the sum of the individual rates. If one rate is much faster than the others, it can be said that the maximum rate at the

electrode is defined by the slowest step in the fastest scheme.

In studying the electrochemical behavior of enzyme systems, it would be of considerable interest if maximum rates could be assigned and this can be done by assigning a maximum rate to one or more steps in each of the possible reaction schemes. If the maximum overall rate, defined thereby is too low to be of practical significance, further consideration of the direct participation in electrode reactions by enzyme molecules could be abandoned. Alternatively such considerations could shed some light on the conditions (such as concentration) which would have to be attained in order for the electrochemical reaction of enzyme molecules to be of significance.

In the present instance, two possible reaction schemes may be postulated for the purposes of such a treatment. In the first the enzyme either diffuses to and is permanently adsorbed onto the electrode, or is intentionally coated onto the electrode in a separate process. The adsorbed enzyme then reacts with substrate which diffuses in from the solution, to form reduced enzyme, subsequently being regenerated by electron transfer to the electrode, while the substrate oxidation products diffuse away. In scheme two, the enzyme is reduced by reaction with the substrate in the bulk of solution, the reduced enzyme then diffuses to the electrode, is adsorbed, reoxidized by electron transfer to the electrode, desorbed as unreduced enzyme and finally diffuses away. Both schemes can be represented by the series of steps 1 to 6.

Theoretically these two schemes could proceed independently. However, if the electrode surface is covered to a large extent by an adsorbed layer of enzyme, as required by scheme one, it would tend to retard scheme two by preventing access to the surface by reduced enzyme from the solution. On the other hand if no adsorbed enzyme layer is present, scheme one cannot occur, and only the second is possible. At intermediate conditions, both would proceed at reduced rates and it is therefore reasonable to postulate maximum rates as those obtained when one scheme applies to the exclusion of the other.

Prediction of maximum rates for some of the pertinent steps of these two reaction schemes is possible. For the first scheme, step 3 is a promising point of attack, and requires only the assumption that the enzyme activity is the same in the adsorbed phase as in the bulk of the solution. For the second scheme, the rate of step 1, i.e. the diffusion rate of reduced enzyme from the solution to the electrode surface is amenable to approximation.

Calculation of the maximum rate for step 3 of the first scheme is as follows.

Assume an enzyme molecule of dimensions corresponding to a molecular weight of 100,000 to 115,000, of cylindrical configuration. The diameter, d, for a molecule of this nature would be in the range of 60 to 80 Å depending upon the length of the cylinder. Taking the lower diameter and assuming a close packing of the adsorbed enzyme on the electrode surface with one end of the cylinder adsorbed to the electrode and the prosthetic group at the exposed face, the number of molecules adsorbed on unit area of the electrode is simply $1/d^2$ and moles of enzyme is

$$N = \frac{i}{d^2(6.02 \times 10^{23})}$$

Further, assume that the overall rate of the complete reaction (enzyme reduction by substrate and oxidation by the electrode) is similar to that occurring at maximal conditions in free solution. The magnitude of the electric current then becomes:

$$I = nNTF$$

where I = current in coulomb sec^{-1}; N = number of moles of enzyme per unit surface; F = 96,500 coulombs; T = turnover number of the enzyme in moles per mole per second, and n = number of electrons per mole.

For D-amino acid oxidase, T = 10 at 25° when saturated with oxygen and substrate. Therefore, for an enzyme similar to DAO

$$I = \frac{10 \times 96{,}500}{(60 \times 10^{-8})^{2} \times 6.02 \times 10^{23}} = 5.8 \ \mu amp/cm^2$$

Higher temperatures, higher turnover numbers or smaller enzyme molecules would increase these outputs. In particular, there is a possibility that higher turnover numbers should be used for adsorbed enzyme than for enzyme in solution due to effects of orientation of the enzyme at the surface. Also, the tacit assumption used in the calculation that electron transfer is possible only from a single layer of enzyme on the surface may not be valid since mechanisms may exist by which electron transfer through multilayers of biological material is possible. However, these points are presently undetermined. It is thus possible merely to conclude that if the first reaction scheme described above correctly represents the reaction of enzymes at an electrode, and if the assumptions used in the estimate are valid then obtainable currents are not likely to exceed the order of 5 $\mu amp/cm^2$.

A maximum rate has not been calculated for the case of reaction scheme 2, since the needed information on diffusivity of enzyme molecules has not become available. This problem will be treated in future work. It is possible that higher currents can be obtained on the basis of the second scheme. However, one tentative conclusion derivable from the foregoing considerations is that in experimentally looking for such currents it will be necessary to examine enzyme systems which do not become permanently adsorbed on the electrode surface.

SECTION 6

OUTLINE OF WORK FOR REMAINING QUARTER

1. Assembly of the micro-cell and elimination of operational problems will be completed. Performance will be tested by means of observations on reference materials. A few comparison studies will be carried out on biological systems and under condition for which data have been obtained in previous work using the old cells. The data will be compared and used to evaluate the performance of the micro-cell.

2. D-amino acid oxidase will be tested in the micro-cell at enzyme concentrations in the range of 10^{-4} to 10^{-3} M for ability to produce a current by direct reaction between the reduced enzyme and the electrode. If activity is obtained, chronopotentiometric techniques will be applied to a study of the nature of the reaction process.

3. The sulfite-nitrite reductase system will be subjected to further study for possible electrochemical activity in excess of that which might be expected through simple production of hydrogen sulfide or ammonia. Maintenance of a continuous reaction in this system requires some means for re-reduction of the co-enzyme after its oxidation by the NO_2^- or $SO_3^=$. It is proposed to provide such means in the form of a second enzyme-substrate system (specifically glucose-6-phosphate

dehydrogenase and glucose-6-phosphate). It will be necessary to examine the possibility of spurious results due to the presence of this second system.

4. Studies will be continued on model macromolecules for simulating the electrochemical behavior of enzyme systems. Emphasis will be on:

   (a) the feasibility of diazo coupling between toluylene blue and tyrosine or tyrocidin,

   (b) the effect of the coupling on redox characteristics of the coupled dye.

5. Studies will be continued on the maximum rate for direct oxidation of enzyme molecules at an electrode. Emphasis will be on such rates based on the diffusivity of enzyme molecule through a solution (steps 1 and 6, Section 4).

REFERENCES

1. R.B. Roberts, P. H. Abelson, D. B. Cowil, E. J. Bolton and R. J. Britten, "Studies of Biosynthesis in E. Coli", Carnegie Inst. of Washington, Publication 607, Washington, D.C., 1955.
2. V. Massey, G. Palmer and R. Bennet, Biochem. Biophys. Acta, 48:1. 1961.
3. Magna Products Inc., Technical Reports, Project BEEP, Contracts NObs 78659, NObs 84243. 1960-63.
4. J. D. Kemp, D. E. Atkinson, A. Ehret and R. A. Lazzarini, J. Biol. Chem., 238:3466. 1963.
5. R. A. Lazzarini and D. E. Atkinson, J. Biol. Chem., 236:3330. 1961.
6. J. J. Lingane and S. L. Jones, Anal. Chem., 23:1798. 1951.
7. D. G. Davis, Anal. Chem., 35:764. 1963.
8. R. C. Valentine, L. E. Mortenson and J. E. Carnahan, J. Biol. Chem, 238:1141. 1963.
9. K. Tagawa, H. Y. Tsujimoto and D. L. Arnon, Proc. Nat. Acad. Sci., 49:569. 1963.
10. K. Tagawa and D. I. Arnon, Nature, 195:537. 1962.
11. B. B. Buchanan, W. Lovenberg and J. C. Rabinowitz, Proc. Nat. Acad. Sci., 49:345, 1963.
12. H. G. Cassidy, et al, J. Am. Chem. Soc., 75:1615. 1953.
    H. G. Cassidy, J. Am. Chem. Soc., 71:402. 1949.
13. C. G. Overberger and A. Lebovits, J. Am. Chem. Soc., 77:3675. 1955.
    H. P. Gregor, D. Dolan and G. K. Hoeschele, J. Am. Chem. Soc., 77:3675. 1955.
14. K. Landsteiner, "The Specificity of Serological Reactions", 2nd Ed., Harvard University Press, Cambridge, 1945.

DISTRIBUTION

National Aeronautics and
Space Administration
Washington, D. C. 20546

Attn: Miss Millie Ruda,
Code AFSS-LD — 1 Copy
Walter C. Scott,
Code RPP — 1 Copy
Ernst M. Cohn,
Code RP — 1 Copy
Dr. Michael Del Duca,
Code RBB — 1 Copy
Dr. W. A. Mensel,
Code REI — 1 Copy
Dr. G. J. Jacobs,
Code SA — 1 Copy
Dr. R. Miles,
Code SL — 1 Copy
A. M. Andrus,
Code FC — 1 Copy
George F. Esenwein,
Code MSA — 1 Copy
John L. Sloop,
Code RP — 3 Copies

National Aeronautics and
Space Administration
Ames Research Center
Moffett Field, California 94035

Attn: Dr. Charles H. Fugitt — 1 Copy
A. S. Hertzog — 1 Copy

National Aeronautics and
Space Administration
Lewis Research Center
21000 Brookpark Road
Cleveland, Ohio 44135

Attn: N. D. Sanders — 1 Copy
M. J. Saari — 1 Copy
R. L. C
B. T. Lubarsky — 1 Copy
Meyer R. Unger 86-1 — 1 Copy + 1 Repro
Harvey J. Schwartz 10X10 SWT
Rm 101
(Procurement Officer)
(LeRC Programs) — 1 Copy

National Aeronautics and
Space Administration
Manned Space Craft Center
Houston, Texas 77001

Attn: E. J. Meeks
(Crew Systems Division) — 1 Copy

National Aeronautics and
Space Administration
Western Operations Office
150 Pico Boulevard
Santa Monica, California 90406

Attn: J. P. Cullinane — 3 Copies
P. Pomerantz — 1 Copy

National Aeronautics and
Space Administration
Office of Scientific and
Technical Information
Washington, D. C. 20546

Attn: AFSS-A — 1 Copy + 1 Repro

U. S. Army Electronics R and D Labs
Fort Belvoir, Virginia

Attn: Mr. B. C. Almaula
Electrical Power Branch — 1 Copy

U. S. Army Engineer R and D Labs.
Fort Monmouth, New Jersey

Attn: David Linden
Code SELRA/SL-PS — 1 Copy
Dr. Adolph Fischbach
Code SELRA/SL-PS — 1 Copy
Arthus F. Daniel
Code SELRA/SL-PS — 1 Copy

U. S. Army R and D Liaison Group
(9851 DV)
APO 757
New York, New York

Attn: Chief, Chemistry Branch — 1 Copy

U. S. Army Limited War Lab.
Aberdeen Proving Ground
Aberdeen, Maryland

Attn: Col. Joseph T. Brown — 1 Copy

U. S. Army Signal R and D Lab.
Power Sources Division
Fort Monmouth, New Jersey

Attn: Dr. Herbert Hunger — 1 Copy

U. S. Army Research Office
Physical Sciences Division
3045 Columbia Pike
Arlington, Virginia

Attn: Dr. Sidney J. Magram — 1 Copy

Harry Diamond Labs.
Room 300, Building 92
Conn. Ave and Van Ness St. N.W.
Washington, D. C.

Attn: Robert Goodrich — 1 Copy

Army Materiel Command
Research Division
AMCRD-RSCM T-7
Washington 25, D. C.

Attn: John W. Crellin — 1 Copy

Natick Labs.
Clothing and Organic Materials
Division
Natick, Massachusetts

Attn: Leo A. Spano — 1 Copy
Robert N. Walsh — 1 Copy

U. S. Army TRECOM
Physical Sciences Group
Fort Eustis, Virginia

Attn: Dr. R. L. Echols (SMOFE-PSG) — 1 Copy
Leonard M. Bartone (SMOFE-ADS) — 1 Copy

U. S. Army Research Office
Box CM, Duke Station
Durham, North Carolina

Attn: Paul Greer — 1 Copy
Dr. Wilhelm Jorgensen — 1 Copy

U. S. Army Mobility Command
Research Division
Center Line, Michigan

Attn: O. Renius (AMSMO-RR) — 1 Copy

Hq., U. S. Army Materiel Command
Development Division
Washington 25, D. C.

Attn: Marshall D. Aiken (AMCRD-DE-MO-P) — 1 Copy

Office of Naval Research
Department of the Navy
Washington 25, D. C.

Attn: Dr. Ralph Roberts 1 Copy
Dr. J. C. White 1 Copy
H. W. Fox 1 Copy
Code 425

Bureau of Naval Weapons
Department of the Navy
Washington 25, D. C.

Attn: Whitwell T. Beatson
Code RAAE-52 1 Copy
Milton Knight
Code RAAE-511 1 Copy

Office of Naval Research
Department of the Navy
Microbiology Branch (Code 443)
Washington 25, D. C. 1 Copy

Bureau of Ships
Department of the Navy
Washington 25, D. C.

Attn: Bernard B. Rosenbaum
Code 340 1 Copy
C. F. Viglotti
Code 660 1 Copy
James B. Trout
Cdr. Joseph W. Thornbury
Code 649 1 Copy

Naval Ordnance Laboratory
Department of the Navy
Corona, California

Attn: Mr. William C. Spindler
Code 441 1 Copy

Naval Ordnance Laboratory
Department of the Navy
Silver Spring, Maryland

Attn: Philip B. Cole
Code WB 1 Copy

Wright Air Development Division
Wright-Patterson Air Force Base, Ohio

Attn: WWRMFP-2 1 Copy

Aeronautical Systems Division
Wright-Patterson Air Force Base, Ohio

Attn: George W. Sherman 1 Copy
Robert L. Kerr 1 Copy
James E. Cooper 1 Copy

AF Cambridge Research Lab
Attn: CRZE
L. G. Hanscom Field
Bedford, Massachusetts

Attn: Francis X. Doherty 1 Copy
Edward Raskind (Wing F)
1 Copy
Commander (CRO) 1 Copy

Rome Air Development Center, ESD
Griffiss AFB, New York

Attn: Commander (RAALD)
1 Copy
1 Copy

Hq., USAF (AFRST-PM)
Washington 25, D. C.

Attn: Lt. Col. William G. Alexander
1 Copy

Capt. William H. Ritchie
Space Systems Division
Air Force Unit Post Office
Los Angeles 45, California

Attn: SSZAE-11 1 Copy

Capt. William Hoover
Air Force Ballistic Missile Division
Air Force Unit Post Office
Los Angeles 45, California

Attn: WEZYA-21 1 Copy

Mr. Charles F. Yost
Asst. Director, Material Sciences
Advanced Research Projects Agency
The Pentagon, Room 3E 153
Washington 25, D. C. 1 Copy

Dr. John Huth
Advanced Research Projects Agency
The Pentagon, Room 3E 157
Washington 25, D. C. 1 Copy

U. S. Atomic Energy Commission
Auxiliary Power Branch (SNAP)
Division of Reactor Development
Washington 25, D. C. 1 Copy

Lt. Col. John H. Anderson
Advanced Space Reactor Branch
Division of Reactor Development
U. S. Atomic Energy Commission
Washington 25, D. C. 1 Copy

Mr. Donald B. Hoatson
Army Reactors, DRD
U. S. Atomic Energy Commission
Washington 25, D. C. 1 Copy

Defense Documentation Center
Headquarters
Cameron Station, Bldg. 5
5010 Duke Street
Alexandria 4, Virginia

Attn: TISIA 1 Copy

Eastern Experiment Station
Bureau of Mines
College Park, Maryland

Attn: Mr. Joseph Sutton 1 Copy

Dr. H. E. Rosvold
Chief, Section on Neuropsychology
National Institute of Mental Health
Bethesda 14, Maryland 1 Copy

Power Information Center
University of Pennsylvania
Moore School Building
200 South 33rd Street
Philadelphia 4, Pennsylvania
1 Copy

Institute for Defense Analyses
1666 Connecticut Avenue, N. W.
Washington 9, D. C.

Attn: Dr. Leon M. Sharpe 1 Copy

Ford Motor Company
Aeronutronic Division
Newport Beach, California

Attn: Dr. Sol W. Weller 1 Copy

Electro-Optical Systems, Inc.
170 N. Daisy Avenue
Pasadena, California

Attn: Dr. Joseph Newstein 1 Copy

Flow Laboratories, Inc
1800 N. Moore Street, Room 402
Arlington, Virginia

Attn: Dr. John A. Welsh 1 Copy

General Electric Company
Electronics Laboratory
Syracuse, New York

Attn: Dr. W. J. Van der Grinten
1 Copy

DISTRIBUTION (Continued)

General Electric Company
Space Sciences Laboratory
King of Prussia, Pennsylvania

Attn: Dr. John J. Konikoff 1 Copy

General Motors Corporation
Box T
Santa Barbara, California

Attn: Dr. C. R. Russell 1 Copy

General Scientific Corporation
2503 Tracy Place, N. W.
Washington, D. C.

Attn: Dr. Robert I. Sarbacher 1 Copy

Leesona Moos Laboratory
Lake Success Park
Community Drive
Great Neck, New York

Attn: Dr. A. M. Moos 1 Copy

Magna Products, Inc.
1001 S. East Street
Anaheim, California

Attn: Dr. Gilson H. Rohrback 1 Copy

The Marquardt Corp.
16555 Saticoy Street
Van Nuys, California

Attn: Mr. George Ellis 1 Copy

Evans Research and Development Co.
250 East 43rd Street
New York 17, New York

Attn: Norman Oxnam 1 Copy

University of Pennsylvania
Electrochemistry Laboratory
Philadelphia 4, Pennsylvania

Attn: Prof. John O'M. Bockris 1 Copy

Rutgers University
Department of Agricultural Microbiology
Nichol Avenue
New Brunswick, New Jersey

Attn: Dr. Robert L. Starkey 1 Copy

Washington University
Adolphus Busch III Laboratory
St. Louis 30, Missouri

Attn: Prof. Howard Gest 1 Copy

Union Carbide Corporation
Parma Research Center Library
P. O. Box 6116
Cleveland 1, Ohio 1 Copy

Institute for Direct Energy Conversion
University of Pennsylvania
Philadelphia 4, Pennsylvania

Attn: Dr. Manfred Altman
Director 1 Copy

Midwest Research Institute
425 Volker Boulevard
Kansas City 10, Missouri

Attn: Dr. David Fischer — 1 Copy

Melpar, Inc
3000 Arlington Blvd
Falls Church, Virginia

Attn: Dr. R. T. Foley — 1 Copy
Dr. Howard Elakman — 1 Copy

The Franklin Institute
Philadelphia, Pennsylvania

Attn: Mr. Robert Goodman — 1 Copy

Dr. Robert Tischer
Mississippi State University
Department of Microbiology
Box 296
State College, Mississippi
1 Copy

Mr. Vernon E. Adler
Contracts Manager
Materials Research Corporation
Orangeburg, New York
1 Copy

Mr. C. S. Coe
AiResearch Manufacturing Company
9851 Sepulveda Blvd
Los Angeles 9, California
1 Copy